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Journal of Neurochemistry, 2001, 77, 638–646

Compensatory mechanisms enhance hippocampal acetylcholine release in transgenic mice expressing human acetylcholinesterase

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Abstract

Central cholinergic neurotransmission was studied in learning-impaired transgenic mice expressing human acetylcholinesterase (hAChE-Tg). Total catalytic activity of AChE was approximately twofold higher in synaptosomes from hippocampus, striatum and cortex of hAChE-Tg mice as compared with controls (FVB/N mice). Extracellular acetylcholine (ACh) levels in the hippocampus, monitored by microdialysis in the absence or presence of 10^{-8}–10^{-3} M neostigmine in the perfusion fluid, were indistinguishable in freely moving control and hAChE-Tg mice. Muscarinic receptor functions were unchanged as indicated by similar effects of scopolamine on ACh release and of carbachol on inositol phosphate formation. However, when the mice were anaesthetized with halothane (0.8 vol. %), hippocampal ACh reached significantly lower levels in AChE-Tg mice as compared with controls. Also, the high-affinity choline uptake (HACU) in hippocampal synaptosomes from awake hAChE-Tg mice was accelerated but was reduced by halothane anaesthesia. Moreover, hAChE-Tg mice displayed increased motor activity in novel but not in familiar environment and presented reduced anxiety in the elevated plus-maze test. Systemic application of a low dose of physostigmine (100 μg/kg i.p.) normalized all of the enhanced parameters in hAChE-Tg mice: spontaneous motor activity, hippocampal ACh efflux and hippocampal HACU, attributing these parameters to the hypocholinergic state due to excessive AChE activity. We conclude that, in hAChE-Tg mice, hippocampal ACh release is up-regulated in response to external stimuli thereby facilitating cholinergic neurotransmission. Such compensatory phenomena most likely play important roles in counteracting functional deficits in mammals with central cholinergic dysfunctions.

Keywords: acetylcholine, cholinergic dysfunction, hAChE-transgenic mice, high-affinity choline uptake, mice, microdialysis.

J. Neurochem. (2001) **77**, 638–646.

Compensatory mechanisms in the CNS may be involved in the extended preclinical stages that are often observed in neurodegenerative diseases, e.g. Parkinson's and Alzheimer's disease (Zigmond 1997). For example, symptoms of Parkinson's disease emerge only when the number of dopaminergic neurons in the substantia nigra is reduced by 80% (Zigmond *et al.* 1990). However, the compensatory capacity of cholinergic neurons for up-regulating cholinergic hypofunction in the brain is not yet known. Central cholinergic dysfunction is associated with cognitive impairment. In humans, this is reflected by the central cholinergic dysfunction associated with Alzheimer's disease and the palliative value of acetylcholinesterase (AChE) inhibitors in this condition (reviewed by Winkler *et al.* 1998). Animal models of cognitive impairment have been established which involve cholinergic imbalance of various kinds, such as acute surgical or chemical lesioning of cholinergic pathways (Schliebs *et al.* 1996) and genetic loss of cholinergic forebrain neurons due to strain differences (Bentivoglio *et al.* 1994; Sago *et al.* 1998). In addition, a unique mouse model displaying an age-dependent, progressive cognitive dysfunction was established by inducing transgenic expression of human AChE (hAChE) limited to brain neurons normally

Received November 2, 2000; revised manuscript received December 21, 2000; accepted January 12, 2001.

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Abbreviations used: ACh, acetylcholine; AChE, acetylcholinesterase; hAChE-Tg, human acetylcholinesterase-transgenic; HACU, high-affinity choline uptake.

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expressing murine AChE (Beeri *et al.* 1995; Andres *et al.* 1996). The overall catalytic activity of AChE in hAChE-Tg mouse brains was found to be enhanced by twofold. The hypothermic effects of AChE inhibitors and cholinoceptor agonists were suppressed in these mice (Beeri *et al.* 1995; Andres *et al.* 1996) and spatial learning, as tested in the Morris water maze, was progressively impaired. These changes were accompanied by elevated [^{3}H]hemicholinium binding in cholinergic target regions in the brains of transgenic mice (Beeri *et al.* 1997), suggesting an increase of the high-affinity choline uptake (HACU) protein level.

The present study investigates compensatory mechanisms of cholinergic neurons in the hippocampus of hAChE-Tg mice. These transgenic mice are excellently suitable for this study since they are suffering from cholinergic hypofunction from birth on. Because they displayed delayed, progressive behavioural and cognitive impairments we chose to investigate the hippocampus due to its established role in learning and memory. Therefore, we took the challenge to adapt the microdialysis method to mouse hippocampus to monitor extracellular ACh levels. Unexpectedly, we observed identical ACh levels in the hippocampi of alert control and hAChE-Tg mice; combined biochemical analysis, microdialysis and behavioural tests demonstrated that compensatory mechanisms are responsible for the normalization of hippocampal ACh levels. Our results reveal a significant capacity for adaptive phenomena of cholinergic neurons to compensate for central cholinergic dysfunction.

Materials and methods

Materials

[^{3}H]Choline and [^{3}H]*myo*-inositol were from DuPont-NEN (Bad Nauheim, Germany). Neostigmine, physostigmine, carbachol, scopolamine, hemicholinium-3 and nicotine were from Sigma Co. (Deisenhofen, Germany) and halothane (Fluothane®) from Zeneca (Plankstadt, Germany), all in the highest quality available.

Animals

Human acetylcholinesterase-transgenic mice carrying the human AChE coding sequence under the control of 586 bp of the authentic human AChE promoter were generated by injection of human AChE constructs into fertilized eggs of FVB/N mice as described before (Beeri *et al.* 1995). For the present studies, we selected animals at an age of 5–7 months which were shown to be cognitively impaired (Beeri *et al.* 1995, 1997). FVB/N mice of the same age were used as controls. The transgenic genotype of individual animals was verified by PCR analysis of tail DNA (Beeri *et al.* 1995, 1997).

The experimental procedures used in this study met the guidelines of and were approved by the responsible governmental agency (Bezirksregierung Rheinland-Pfalz).

Microdialysis

Mice were anaesthetized with pentobarbital (60–80 mg/kg) and placed in a stereotactic frame. I-shaped, miniature, concentric dialysis probes with an exchange length of 1 mm were constructed and implanted in the dorsal mouse hippocampus using the following coordinates (from bregma): AP −2.0 mm; L −1.8 mm; DV −2.3 mm (cf. Fig. 1). Experiments were carried out 1 or 2 days after probe implantation in freely moving or anaesthetized animals. In some cases, light anaesthesia was accomplished with halothane (0.8 vol. %, controlled by halothane vaporizer) in a special container which allowed microdialysis to be carried out during halothane inhalation. Probes were perfused with artificial cerebrospinal fluid (CSF) (147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$) containing neostigmine and/or scopolamine or nicotine (see Results) at a perfusion rate of 1 μL/min; the *in vitro* recovery for ACh was determined as 11.6 ± 1.4% ($N = 4$). At the end of experiments, the brains were fixed by transcardial perfusion with 4% phosphate-buffered paraformaldehyde solution (pH = 7.4) and stained for AChE activity (Koelle 1954; modified as described in Erb *et al.* 1997) to confirm the location of the probe.

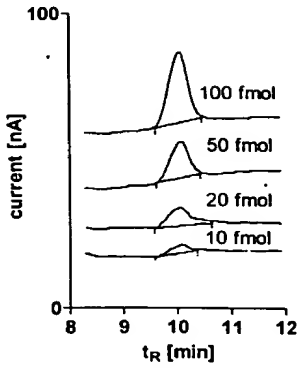

Fig. 1 Detection of ACh by microbore HPLC in dialysate of mouse hippocampus. For details see Materials and methods.

Determination of ACh

ACh in dialysates was determined by microbore HPLC using an optimized, metal-free system which consisted of a low-speed pump (BAS PM80), separation column (SepStik, 530 × 1 mm), enzyme reactor (50 × 1 mm) carrying immobilized AChE and choline oxidase and electrochemical detector (BAS LC-4C) with a platinum electrode operating at 0.5 V. The flow rate was 120 μL/min. At an injection volume of 5 μL, the detection limit of this system was 10–20 fmol/injection (Fig. 1).

Synaptosomes and AChE activity

Synaptosomes were prepared from hippocampal, striatal and cortical tissue according to Dunkley *et al.* (1988). Brain tissue was homogenized in isotonic sucrose solution (0.32 M, 1 mM EDTA, 0.25 mM dithiothreitol, pH 7.4) and centrifuged at 1000 *g* at 4°C for 10 min. The supernatant was layered on top of a discontinuous Percoll gradient (3%, 10% and 23%) and centrifuged at 32 500 *g* at 0°C for 10 min. The synaptosomal fraction was recovered between the 10 and 23% layers and washed three times in 0.1 M phosphate buffer (pH 7.4). Subsequently, synaptosomal AChE activity was determined (Ellman *et al.* 1961) and was expressed as the amount of acetylthiocholine iodide (out of 1 mM) hydrolyzed by one mg synaptosomal protein in 1 min of incubation

in the presence of 0.5 mM 5,5′-dithio-*bis*-dinitro-benzoate (DTNB). Protein content was determined by the Lowry procedure.

High-affinity choline uptake (HACU)

HACU was determined in P_2 fractions obtained from hippocampus, cortex and striatum essentially as described by Cassel *et al.* (1992). Brain tissue was homogenized in isotonic sucrose solution and centrifuged at 1000 *g* at 4°C for 10 min as described above. Subsequently, the supernatant was again centrifuged at 17 000 *g* for 10 min, and the resulting P_2 pellet was washed and used for HACU determinations. For this purpose, aliquots were incubated with 50 nM [^{3}H]choline at 30°C for exactly 5 min. Choline uptake was stopped by addition of ice-cold buffer. After centrifugation, the pellet was washed three times with sodium-free buffer, and radioactivity associated with the pellet was determined by liquid scintillation counting. HACU was expressed as [^{3}H] uptake (dpm/mg protein/5 min) after subtraction of nonspecific uptake determined in parallel incubations in the presence of 1 μM hemicholinium-3.

Determination of inositol phosphates

The formation of total inositol phosphates was determined in hippocampal slices (400 μm) essentially as described (Klein *et al.* 1997). Briefly, slices were incubated with 40 μCi [^{3}H]*myo*-inositol for 30 min in a buffer containing 10^{-4} M di-isopropyl-fluorophosphate (DFP) to inhibit AChE. Subsequently, the slices were superfused for 20 min with buffer containing 10 mM lithium chloride and then with carbachol (0.01–1 mM) for 60 min. After homogenization in chloroform/methanol, the aqueous phase was transferred to a BioRad AG 1-X8 anion exchange column, and total inositol phosphates were eluted by 60 mM formate. Inositol phosphate formation was expressed as percentage of total of [^{3}H]inositol labelling incorporated into the slices.

Behavioural experiments

Locomotor activity was monitored in the open field cage (30 × 50 cm) with a floor divided into six squares and expressed as the frequency of line crossings per 10 min. To measure exploration activity, mice were exposed to a novel object 5 min after the beginning of the open field session, and exploration was expressed as 'time spent exploring the novel object'. Anxiety-related behaviour was analyzed as described (Schmitt and Hiemke 1998) in the elevated plus-maze (open arms 47.5 × 5 cm; closed arms 47.5 × 5 × 28 cm; central platform 5 × 5 cm) and registered automatically by a computerized image analysis system (EthoVision; Noldus Information Technology, Wageningen, the Netherlands). The parameters recorded were 'total distance moved on open/closed arm', 'number of closed/open arm entries' and 'time spent on open arm'.

Results

Synaptosomal AChE activity

In synaptosomes from hippocampus, striatum and cortex prepared from FVB/N mice, we found that basal AChE activity was highest in the striatum and lowest in the hippocampus (Fig. 2). In hAChE-Tg mice, the synaptosomal AChE activity was almost doubled in all three selected brain regions as compared with controls (Fig. 2). Thus, hAChE-expression enhanced AChE activity in the synaptic region.

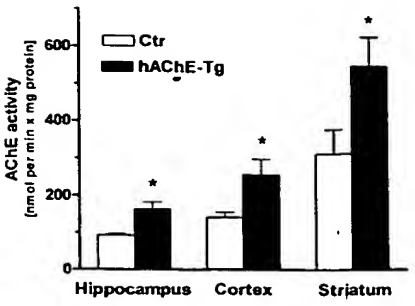

Fig. 2 AChE activity in synaptosomes from hippocampus, cortex and striatum of control and hAChE-Tg mice. Synaptosomes were prepared from the brain regions of 5–7-month-old mice. AChE activity determined according to Ellman *et al.* (1961) was expressed as nmol acetylthiocholine hydrolyzed per min × mg protein. Presented are means ± SEM of 4–5 experiments. *Significantly different from control ($p < 0.05$).

Extracellular ACh concentration in the hippocampus of freely moving mice

Microdialysis is best suited for studying ACh release under cholinergic hypofunction *in vivo*. Therefore, we adapted the design of the microdialysis probe and the stereotactic coordinates for implantation into the mouse hippocampus and improved ACh analysis (cf. Figure 1). When increasing concentrations of neostigmine (10^{-8}–10^{-3} M) were added to the perfusion medium, ACh efflux in the mouse hippocampus increased (Fig. 3; see also Moor *et al.* 1998). It should be noted, however, that 10^{-8} and 10^{-7} M neostigmine failed to enhance basal ACh levels, a finding which likely reflects the presence of low but measurable extrasynaptic levels of ACh (Descarries 1998). Importantly, ACh efflux was identical in control and in transgenic mice (Fig. 3).

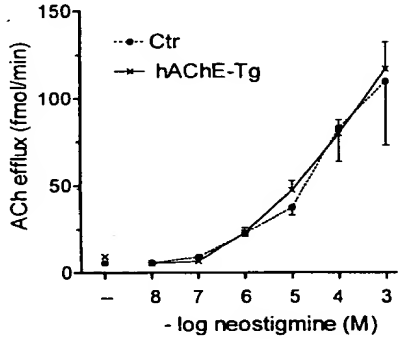

Fig. 3 ACh efflux from the dorsal hippocampus of freely moving control and hAChE-Tg mice. Note that ACh efflux was identical in control and hAChE-Tg mice in the absence or presence of neostigmine (10^{-8}–10^{-3} M) added to the perfusion medium. Presented are means ± SEM of 4–11 experiments.

There was no indication of suppressed ACh levels, either in the absence or presence of neostigmine, and no evidence for a shift of the curve to the right which we had expected from the overexpression of catalytically active AChE in transgenic animals (Fig. 2).

Pre- and postsynaptic cholinergic mechanisms in the hippocampus

To test for possible adaptive changes of presynaptic receptor function in AChE-Tg mice, we infused scopolamine into the hippocampus to block presynaptic muscarinic receptors. In the presence of 10^{-5} M neostigmine, scopolamine increased the basal ACh efflux in control (+570 ± 80%, $N = 7$) and transgenic mice (+600 ± 95%, $N = 6$) alike (Fig. 4a); time-course and maximum effect did not differ significantly between the groups. This scopolamine-induced increase reflected blockade of muscarinic autoinhibition caused by neostigmine which reduced ACh release by 83% in both groups of animals under the present conditions (calculated from the six-fold increase of ACh in Fig. 4a). In separate experiments, infusion of nicotine (300 μM) into the hippocampus did not change the basal efflux of ACh (23 fmol/min) into the microdialysis perfusate containing 10^{-6} M neostigmine, either in control or in transgenic mice (not documented).

To examine possible changes of postsynaptic receptor function in AChE-Tg mice, we measured the formation of inositol phosphates in hippocampal slices taken from control and transgenic animals. Stimulation of muscarinic receptors by 10^{-4}, 10^{-3} and 10^{-2} M carbachol enhanced the formation of [^{3}H]inositol phosphates (expressed as percentage of total [^{3}H]inositol labelling) in control mice and, to a similar extent, in transgenic mice (Fig. 4b). Atropine (1 μM) blocked the responses to carbachol in both groups (data not shown).

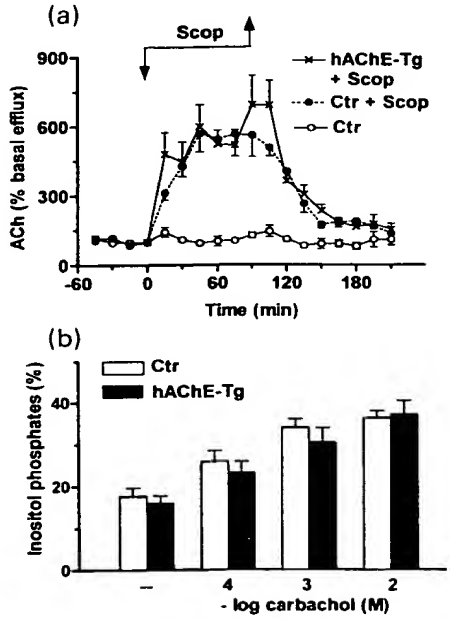

Fig. 4 Pre- and postsynaptic cholinergic effects in the hippocampus of control (Ctr) and hAChE-Tg mice. (a) Effect of 10^{-6} M scopolamine in the perfusion fluid on ACh efflux into the perfusate containing 10^{-5} M neostigmine; the efflux was expressed as percentage of basal efflux (100% = 37 or 48 fmol/min in control or hAChE-Tg mice, respectively). Scopolamine was infused for 90 min as indicated by the arrows. (b) Effect of carbachol (10^{-4}–10^{-2} M) on the formation of [^{3}H]inositol phosphates in hippocampal slices taken from control and hAChE-Tg mice. Inositol phosphates were expressed as percentage of total lipid label. Presented are means ± SEM of six or seven experiments.

Effects of halothane anaesthesia

We repeated the microdialysis experiments in mice anaesthetized with halothane. Halothane drastically reduces the firing rate in the septo-hippocampal pathway (Ngai *et al.* 1978; Dutar *et al.* 1986) but causes only a minor direct attenuation of synaptic ACh release (Damsma and Fibiger 1991). The halothane dose was adjusted to the minimal concentration (0.8 vol. %) that suppressed locomotion and caused loss of righting reflex. During halothane anaesthesia, ACh efflux of the hAChE-trangenic mice was reduced by about 50% compared with control mice at 10^{-6}, 10^{-5} and 10^{-4} M neostigmine (Fig. 5). This was the theoretically expected result for ACh levels in the hippocampus of hAChE-transgenic mice, suggesting that the identical extracellular ACh concentration in awake control and hAChE-transgenic mice (Fig. 3) reflects an accelerated ACh turnover in alert hAChE-transgenic mice.

Changes in the high-affinity uptake of choline

The *in vivo* turnover of ACh is reflected in the hemicholinium-3-sensitive high-affinity choline uptake (HACU)

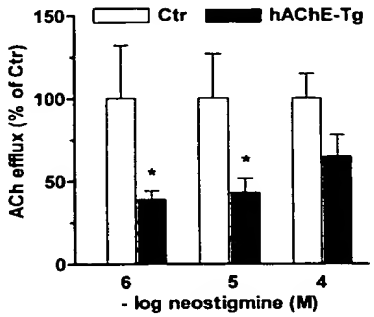

Fig. 5 Hippocampal ACh efflux of control and hAChE-Tg mice during light halothane anaesthesia. ACh levels in hAChE-Tg mice were expressed as percentage of the levels in control mice at three neostigmine concentrations. At 1 μM neostigmine, halothane reduced ACh efflux from 22.7 ± 1.9 (Fig. 3) to 15.0 ± 4.8 fmol/min (= 100%). Presented are means ± SEM of five or six experiments. *Significantly different from control ($p < 0.05$).

measured *ex vivo* (Kuhar and Murrin 1978). We investigated HACU in synaptosomal preparations from different brain regions. Compared to controls, HACU was markedly elevated (+73 ± 21%; $N = 6$) in the hippocampus of awake transgenic mice; a similar increase was observed in the striatum (+58 ± 18%; $N = 6$), but not in the cortex (+3 ± 10%; $N = 11$) (Fig. 6a). Moreover, light halothane anaesthesia which did not significantly affect HACU in control mice reduced the elevated HACU found in freely moving transgenic mice (Fig. 6b).

Effects of physostigmine

After intraperitoneal administration of a low dose of physostigmine sulphate (100 μg/kg), hippocampal ACh efflux was enhanced in control mice but not in transgenic mice (Fig. 7). Moreover, at the same dose, physostigmine reduced the increased synaptosomal HACU observed in the transgenic mice to control levels (Fig. 6b).

Behavioural studies

Human acetylcholinesterase-transgenic mice exhibited enhanced spontaneous locomotor activity in the open field cage; the increase of locomotion occurred upon first exposure (1st day), but not on day 7, i.e. after habituation (Fig. 8). When, after habituation, hAChE-Tg mice were exposed to a novel object, they again showed an increased explorative activity compared with controls (Fig. 8). Interestingly, the novelty-induced locomotor activity of transgenic mice was blocked by the same low dose of physostigmine (100 μg/kg i.p.) which had specific effects in the previous experiments on ACh levels and HACU (Figs 6 and 7).

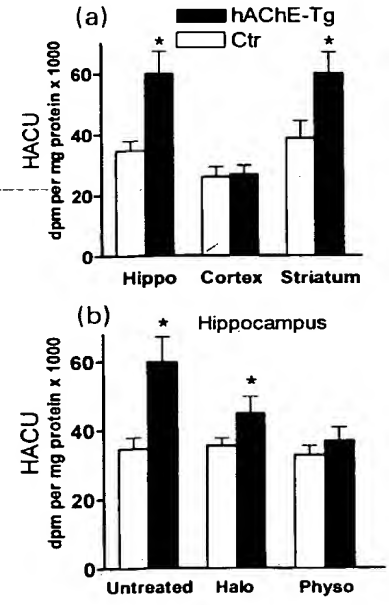

Fig. 6 High-affinity choline uptake (HACU) into synaptosomes from hippocampus, cortex and striatum of control and hAChE-Tg mice. HACU was expressed as the hemicholinium-3-sensitive uptake of [^{3}H]choline per mg synaptosomal protein in 5 min. (a) Synaptosomes prepared from hippocampus (Hippo), cortex and striatum of untreated mice. (b) Hippocampal synaptosomes prepared from untreated mice, from mice in halothane anaesthesia (Halo; 0.8 vol. %; 1 h) and from mice 30 min after intraperitoneal injection of physostigmine (Physo; 100 μg/kg). Presented are means ± SEM of five or six experiments. *Significantly different from control ($p < 0.05$).

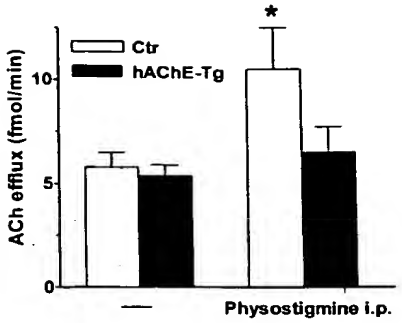

Fig. 7 Effect of systemically applied physostigmine on hippocampal ACh efflux of freely moving control (Ctr) and hAChE-Tg mice. ACh levels were measured 30 min after intraperitoneal injection of 100 μg/kg physostigmine. Neostigmine (10^{-8} M) was added to the microdialysis perfusion medium in all experiments. Presented are means ± SEM of 6–12 experiments. *Significantly different ($p < 0.05$) from untreated control mice.

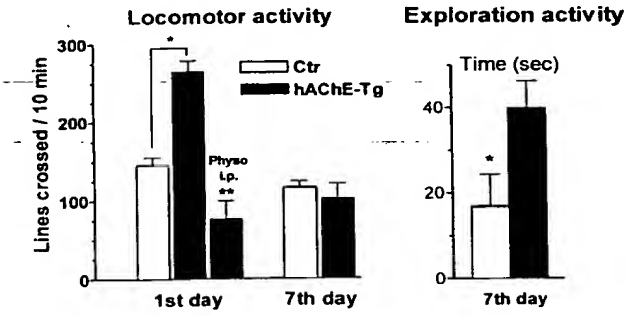

Fig. 8 Locomotor and exploration activities of control (Ctr) and hAChE-Tg mice in the open field test. Left panel: Locomotor activity expressed as lines crossed in 10 min was measured on 7 consecutive days; presented are the observations upon 1st exposure (1st day) and after habituation (7th day). In some transgenic mice, locomotion was determined 30 min after intraperitoneal injection of physostigmine (100 μg/kg). Right panel: Exploration activity was studied by exposing the mice to a novel object in the open field cage and expressed as time (s) spent with the novel object during a total observation period of 300 s. Presented are means ± SEM of 15 (left panel) and nine (right panel) experiments. *Significantly different ($p < 0.05$) from hAChE-Tg mice. **Significantly different ($p < 0.01$) from 1st day locomotion of untreated hAChE-Tg mice.

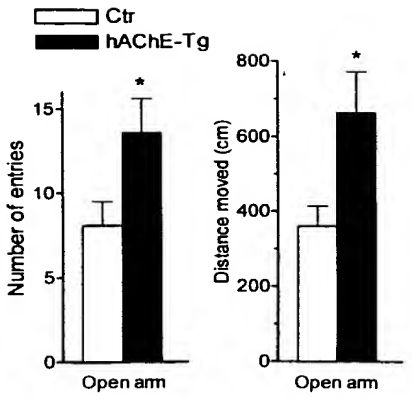

Fig. 9 Anxiety-related behaviour of control (Ctr) and hAChE-Tg mice in the elevated plus-maze test. Left panel: Number of open arms entries. Right panel: Distance moved on the open arms (cm). Determination of other parameters in this test (see Materials and methods) did not reveal any differences between control and hAChE-Tg mice. Presented are means ± SEM of 15 mice per group. *Significantly different ($p < 0.05$) from controls.

The elevated plus-maze test was selected to unravel potential alterations in anxiety-like behaviour. We found that hAChE-Tg mice entered open arms more frequently and moved larger distances on the open arms as compared with controls (Fig. 9). In contrast, the total distance they moved on open/closed arms and their total moving time were similar in control and hAChE-Tg mice (not documented). This suggests that the increased novelty-induced locomotor activity in hAChE-Tg mice was due to reduced anxiety and/or a reduced retreat behaviour.

Discussion

Compensatory mechanisms play important roles in neurodegenerative disease. This has been well described for dopaminergic neurotransmission in Parkinson's, but not yet for cholinergic neurotransmission in connection with Alzheimer's disease. Therefore we were interested to investigate if cholinergic neurons are capable to compensate for cholinergic deficits. We employed hAChE-Tg mice with central cholinergic hypofuntion to investigate compensatory mechanisms in the hippocampus *in vivo*. Our study demonstrates that, in awake rats, hippocampal cholinergic neurons are able to enhance ACh release sufficiently to reach control levels of ACh in the extracellular space.

Unlike other models based on cholinergic dysfunction, but similar to real-life situations, the hAChE-Tg mice develop an age-dependent, progressive deterioration of memory within 6 months from birth (Beeri *et al.* 1995; Andres *et al.* 1996). Therefore, the present studies were carried out using mice at an age of 5–7 months.

Previous investigations using cytochemical staining of catalytically active hAChE revealed that the presence of the human enzyme in these mice was confined to brain regions with cholinergic innervation, where it was coexpressed with murine AChE; intense AChE activity staining was observed in striatum and hippocampus (Beeri *et al.* 1995; Andres *et al.* 1996). By isolating synaptosomes, we now show that catalytically active AChE is markedly enhanced in the nerve terminals of the hippocampus, striatum and cortex, i.e. at the sites of ACh release (Fig. 2). Therefore, our observation that the extracellular levels of ACh in the hippocampus of freely moving hAChE-Tg mice were not reduced as compared with controls was totally unexpected (Fig. 3). In these experiments, ACh levels were determined in the perfusate of a microdialysis probe positioned in the dorsal hippocampus. The hippocampus was chosen as the brain area of interest in this study because of its well established role in learning and memory. With the support of a single microdialysis study which demonstrated ACh in the mouse hippocampus (Imperato *et al.* 1996) we succeeded to adapt the microdialysis technique from the rat to the mouse brain; this effort was justified not only for the present study, but for future studies of cholinergic parameters in gene-manipulated mice.

The surprising finding that hippocampal ACh levels in the hAChE-Tg mice were normal was complemented by the observation that pre- and postsynaptic cholinergic parameters in this brain region were unchanged, too. There were no differences in the scopolamine-evoked ACh efflux into the microdialysate (mediated via M_2-receptors; cf. Rouse *et al.* 1999) and in the carbachol-induced formation of inositol phosphates in the hippocampal slice preparation (mediated via M_1-receptors) (Fig. 4). At this point of the project, where normal cholinergic parameters in the hippocampus contrasted with enhanced synaptic AChE activity and a behavioural pattern characteristic of central cholinergic dysfunction, we began to search for compensatory mechanisms increasing hippocampal ACh to normal levels. We therefore determined the high-affinity choline uptake (HACU) in hippocampal synaptosomes because HACU is increased post mortem when release and turnover of ACh have been enhanced *in vivo* (Kuhar and Murrin 1978; Tucek 1985). Indeed, synaptosomal HACU was found to be elevated in the hippocampus (+73% of control) of awake hAChE-Tg mice; a similar increase was observed in the striatum, but not in the cortex (Fig. 6a). These results are compatible with a report by Beeri *et al.* (1997) describing an enhanced [^{3}H]hemicholinium binding in the striatum of the hAChE-Tg mice.

As these results suggested an accelerated ACh turnover in the hippocampus of hAChE-Tg mice, we repeated the microdialysis experiments in mice anaesthetized with halothane in order to reduce the firing rate of the septo-hippocampal pathway (Dutar *et al.* 1986). For this purpose, we used a home-made device which allowed on-line microdialysis during continuous halothane vapour exposition.

During halothane anaesthesia, the extracellular ACh levels in hAChE-Tg mice were about half of those found in controls at various neostigmine concentrations (10^{-6}–10^{-4} M; Fig. 5); this result was compatible with what we had expected based on the overexpression of synaptic AChE activity. At the same low concentration, halothane also reduced the enhancement of HACU activity in hippocampal synaptosomes from hAChE-Tg mice (from + 73% to + 30% of control; Fig. 6b). It follows from these observations that freely moving hAChE-Tg mice respond to AChE overexpression with a compensatory elevation of hippocampal ACh turnover.

In spite of these compensatory phenomena, hAChE-Tg mice showed clear-cut behavioural features of cholinergic dysfunction in this and previous studies (Beeri *et al.* 1995, 1997). We therefore wondered whether the behavioural effects of hAChE expression could reflect a hypocholinergic state in brain regions outside the hippocampus. In order to test this hypothesis we systemically applied the centrally acting AChE inhibitor, physostigmine, in a low dose which had little effect on cholinergic parameters in control mice. We made the following observations: (i) The enhancement of HACU in hippocampal synaptosomes of hAChE-Tg mice was completely blocked by physostigmine (Fig. 6b). (ii) In microdialysis experiments, physostigmine enhanced hippocampal ACh levels in control mice, but not in hAChE-Tg mice (Fig. 7). It is unlikely that this response to physostigmine was due to a hippocampal site of action because neostigmine – added to the microdialysis fluid in a wide range of concentrations – did not reveal any difference in hippocampal ACh efflux between control and transgenic mice (Fig. 3). It follows that systemically applied physostigmine must have acted outside of the hippocampus. This would tentatively involve a cholinergic pathway controlling the activation of the septohippocampal fibres and which is affected by cholinergic dysfunction. A similar resistance of hAChE-Tg mice to AChE inhibitors was reported for the hypothermic effect of paraoxon (Beeri *et al.* 1995; Andres *et al.* 1996). (iii) Furthermore, physostigmine blocked the novelty-induced increase in explorative locomotor activity of hAChE-Tg mice in the open field (see below).

We found that hAChE-Tg mice exhibited an increased, physostigmine-sensitive, novelty-dependent locomotor activity in the open field (Fig. 8) similar to that seen in healthy animals treated with muscarinic antagonists (Beleslin *et al.* 1986). Likewise, we found signs of reduced anxiety in the elevated plus-maze paradigm (Fig. 9) which were also seen in AF64A-treated mice, another model of cholinergic dysfunction (Lamberty *et al.* 1992). Exposure of the hAChE-Tg mice to the microdialysis cage prior to the experiment represents a novel situation analogous to the above described behavioural test paradigms which unravelled the novelty-induced locomotor activation. Therefore, the enhanced explorative locomotor activity in the hAChE-Tg mice may have contributed to the compensatory increase of hippocampal ACh release in animals freely moving in the microdialysis cage.

What is the mechanism of the compensatory increase of hippocampal ACh turnover? The major source of cholinergic hippocampal pathways is the medial septum/diagonal band of Broca (MS/DB); electrical stimulation of the medial septum leads to drastic increases of ACh release in the hippocampus (Moor *et al.* 1998). Descending GABAergic projections from the hippocampus to the MS/DB form a septo-hippocampal-septal loop representing the topographical basis for feedback control of hippocampal ACh release (Gaykema *et al.* 1991; Tóth *et al.* 1993). This primary feedback loop receives multifold inputs from various brain regions, including cholinergic pathways such as that of the mesencephalic locomotor region (Mathur *et al.* 1997) which partially projects to the MS/DB (Hallanger and Wainer 1988). These neuronal connections explain why a variety of behavioural activities and stimuli, such as stress, pain, and novelty-induced locomotor activation are associated with modulations of hippocampal ACh release (Inglis and Fibiger 1995; Acquas *et al.* 1996; Aloisi *et al.* 1997). We speculate that, in our experiments, up-regulation of hippocampal ACh release was associated with cholinergic dysfunction in extrahippocampal areas such as midbrain and/or cortex; however, more work is required to confirm this hypothesis.

In conclusion, transgenic overexpression of AChE induced an activity-dependent enhancement of hippocampal ACh release presumably *via* an adaptive modulation of the firing rate of the septohippocampal pathway. The results exemplify the compensatory capacity of central cholinergic circuits to normalize transmitter release in certain brain regions. In spite of the compensatory mechanisms, we saw changes in the behaviour of these animals. As indicated by the experiments with physostigmine, these changes are caused by cholinergic hypofunction. The age-dependent deterioration in these mice might therefore mean that young mice manage to fully compensate for the cholinergic imbalance whereas older ones do not. Compensatory elevations of hippocampal ACh synthesis and turnover had previously been reported after partial lesioning of the septohippocampal pathway (Lapchak *et al.* 1991; Leanza *et al.* 1993; Erb *et al.* 1997). Additional compensatory mechanisms which have been observed include adaptations at the synaptic level such as up- or down-regulations of ACh receptors, increase of the vesicular ACh transporter (Ruberg *et al.* 1990), inductions or repressions of cholinergic genes (Kaufer *et al.* 1998; Von der Kammer *et al.* 1998), and processes of synaptic plasticity (Cassel *et al.* 1997) which are known to fail in Alzheimer's disease (Mesulam 1999). In a study in humans, Slotkin *et al.* (1994) demonstrated increases of HACU expression in Alzheimer brains which was of similar magnitude as that observed in hAChE-Tg mice. Thus, the capacity of cholinergic neurons to compensate for loss of neurons may be of

clinical importance in central cholinergic dysfunctions in humans.

Ackn wledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Kl 598/6–1). We are grateful to Michael Hilgert and Marieluise Buchholzer for contributing data to Figs 1 and 3, respectively.

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